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Synaptotagmin-1 functions as the Ca^{2+}-sensor for spontaneous release

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Abstract

Spontaneous ‘mini’ release occurs at all synapses, but its nature remains enigmatic. Here, we show that in murine cortical neurons, >95% of spontaneous release is induced by Ca^{2+}-binding to synaptotagmin-1, the Ca^{2+}-sensor for fast synchronous neurotransmitter release. Thus, spontaneous and evoked release use the same Ca^{2+}-dependent release mechanism. As a consequence, synaptotagmin-1 mutations that alter its Ca^{2+}-affinity alter spontaneous and evoked release correspondingly. Paradoxically, synaptotagmin-1 deletions (as opposed to point mutations) massively increase spontaneous release. This increased spontaneous release remains Ca^{2+}-dependent, but is activated at lower Ca^{2+}-concentrations, and with a lower Ca^{2+}-cooperativity, than synaptotagmin-driven spontaneous release. Thus, in addition to serving as Ca^{2+}-sensor for spontaneous and evoked release, synaptotagmin-1 clamps a second, more sensitive Ca^{2+}-sensor for spontaneous release which resembles the Ca^{2+}-sensor for evoked asynchronous release. Viewed together, these data suggest that synaptotagmin-1 controls both evoked and spontaneous release at a synapse as a simultaneous Ca^{2+}-dependent activator and clamp of exocytosis.

Spontaneous miniature release (‘minis’) was observed in initial recordings of synaptic activity1, and has engaged neuroscientists ever since2-5. Under physiological conditions, every synapse spontaneously releases neurotransmitter quanta at a low frequency. In a typical central neuron, these quanta add up to a sizable signal because thousands of synapses

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are present on the neuron. A major question has been whether spontaneous neurotransmitter release is biologically meaningful, or accidental. Indeed, the very nature of spontaneous release remains unclear — is it truly spontaneous, or regulated[2,3]? Does it operate with the same synaptic machinery as evoked release, or represent a different type of exocytosis that involves a special synaptic vesicle pool[5,6]? Presynaptic mini release produces multiple postsynaptic effects; for example, spontaneous release maintains dendritic spines[7], regulates cerebellar interneuron firing[8], and suppresses local dendritic protein synthesis[9]. Moreover, many neurotransmitters and psychoactive compounds such as endocannabinoids, caffeine, and nicotine modulate spontaneous release by activating presynaptic receptors[10-16], indicating that spontaneous minis perform a biological function. Consistent with this notion, a significant component of spontaneous release appears to be Ca^{2+}-dependent, and may be triggered by presynaptic Ca^{2+}-sparks[17,18]. An additional sizable component of spontaneous minis, however, was found to be Ca^{2+}-independent in at least some experiments[2-4], although the mechanisms involved remain unclear.

Evoked neurotransmitter release is mediated by a synaptic membrane fusion machinery composed of SNARE- and SM-proteins (for 'soluble NSF-attachment protein receptors' and 'Sec1/Munc18-like proteins', respectively), and is controlled by Ca^{2+}-binding to synaptotagmin-1 (Syt1), synaptotagmin-2 (Syt2), or synaptotagmin-9 which perform similar interchangeable functions in evoked release[19-22]. Deletions of SNARE- and SM-proteins block evoked and spontaneous release, suggesting that the generic synaptic membrane fusion machinery mediates both types of release[23-25]. Deletions of Syt1 and Syt2, however, produce increases in spontaneous mini release in Drosophila neuromuscular junctions and in vertebrate central synapses[26-28] (although not in vertebrate autapses[29]). This finding led to the hypothesis that Syt1 is not only the Ca^{2+}-sensor for evoked release, but also a clamp of SNARE-proteins that limits spontaneous release, and that minis are accidental 'leaks' of this clamping function[20,21]. The clamping hypothesis, however, argues against the notion that spontaneous release may be biologically meaningful, because it is difficult to imagine how an accidential by-product of evoked release could control a physiological process. Moreover, the clamping hypothesis fails to explain why at least some mini release is Ca^{2+}-dependent. In the present paper, we explore how Ca^{2+} regulates spontaneous release, and relate this mechanism to the clamping of spontaneous release by synaptotagmins. We demonstrate that Ca^{2+} triggers most spontaneous release, as it does most evoked release, by binding to synaptotagmin, and that deletion of synaptotagmin activates a second Ca^{2+}-sensor for spontaneous release which has a higher apparent Ca^{2+}-affinity than synaptotagmin. As a result, deletion of synaptotagmin leads to a paradoxical increase in spontaneous release mediated by this second Ca^{2+}-sensor that is normally clamped by synaptotagmin.

RESULTS

Spontaneous release is Ca^{2+}-dependent

Using cultured cortical neurons, we measured spontaneous miniature inhibitory postsynaptic currents (mIPSCs) and evoked inhibitory postsynaptic currents (IPSCs) in extracellular medium containing or lacking Ca^{2+}. Removal of extracellular Ca^{2+} only partly depressed the frequency of spontaneous mIPSCs, but almost completely blocked evoked IPSCs (Figs. 1a

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and 1b and data not shown). Thus, as previously shown, spontaneous mini release is less sensitive than evoked release to reductions in extracellular Ca^{2+}-concentration1-4. However, when we pre-incubated cultured neurons with BAPTA-AM, a membrane-permeable Ca^{2+}-chelator, almost all mIPSCs (>95%) were blocked (Figs. 1a, 1b, and Suppl. Fig. S1). Addition of thapsigargin to neurons in Ca^{2+}-free medium strongly enhanced the mini frequency, but again preincubation with BAPTA-AM blocked spontaneous mini release (Figs. 1a and 1b). Caffeine, which acts to increase presynaptic Ca^{2+}-concentrations30, also increased the frequency of spontaneous release (Fig. S2).

As described above, cortical synapses lacking Syt1 exhibit almost no evoked release but a paradoxical increase in spontaneous release27, which is thought to reflect a clamping of SNARE complexes by Syt1 (ref. 20, 21). This increased spontaneous release together with the loss of evoked release can be rescued by expression of exogenous Syt1, and thus is not developmentally induced (Figs. 1c and 1d). However, the Ca^{2+}-requirement of mini release in wild-type synapses raises the question whether the increased minis in Syt1-deficient synapses may also be Ca^{2+}-dependent. Indeed, we found that >95% of mIPSCs in Syt1-deficient synapses were suppressed by BAPTA-AM (Figs. 1c and 1d), demonstrating that minis are Ca^{2+}-triggered, and cannot be due to simple ‘popping’ of SNARE-assembly for fusion.

A potential concern with these experiments is that Ca^{2+} may be required for the viability of neurons and the integrity of synapses. To ensure that the unexpectedly strong suppression of mIPSCs by BAPTA-AM specifically reflects the effect of withdrawing Ca^{2+} on mIPSCs, and is not due to a simple loss of all synaptic function, we measured the readily-releasable pool (RRP) of vesicles in synapses that were pre-incubated with BAPTA-AM. As estimated by application of hypertonic sucrose31, we found no decrease in the size of the RRP, measured as the total synaptic charge transfer induced by hypertonic sucrose, after pre-incubation with BAPTA-AM (Figs. 1e and 1f). This result also eliminated the possibility of defects in postsynaptic $GABA_A$ receptors after treatment with BAPTA-AM.

Spontaneous release in Syt1 KO synapses

The results of Fig. 1 raise several questions. First, are minis in wild-type and Syt1-deficient synapses produced by an identical Ca^{2+}-dependent process that is simply enhanced by the Syt1 KO, or is there a fundamental difference in the Ca^{2+}-triggering of minis between wild-type and Syt1-deficient synapses? Second, do these results equally apply to excitatory and inhibitory synapses? Third, what is/are the Ca^{2+}-sensors for spontaneous mini release?

To address the first two questions, we measured the Ca^{2+}-dependence of spontaneous release in wild-type and Syt1-deficient synapses, in both excitatory and inhibitory synapses (Fig. 2). Cultured cortical neurons from littermate wild-type and Syt1 KO mice were analyzed at increasing concentrations of extracellular Ca^{2+}, and excitatory and inhibitory minis (mEPSCs and mIPSCs, respectively) were monitored in tetrodotoxin after pharmacological isolation. We observed a similar behavior of spontaneous mini release in excitatory and inhibitory synapses, but a dramatic difference between wild-type and Syt1 KO synapses. At all Ca^{2+} concentrations, the mEPSC and mIPSC frequencies were strongly increased in Syt1 KO synapses, with an apparent left shift in the Ca^{2+}-concentration

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dependence (Figs. 2b and 2c). To quantify this, we fitted the results of individual experiments to a Hill function, and determined the apparent Ca^{2+}-cooperativity (Hill coefficient) and apparent Ca^{2+}-affinity (K_d) of extracellular Ca^{2+} for miniature release. Analysis of multiple independent experiments revealed a significant decrease in the Ca^{2+}-cooperativity, and a large increase in the Ca^{2+}-affinity (i.e., a >3-fold decrease in K_d) of spontaneous mini release in Syt1 KO synapses (Figs. 2d and 2e; note that the apparent Ca^{2+}-cooperativity and Ca^{2+}-affinity of release determined by varying the extracellular Ca^{2+}-concentration, as performed for this study, underestimates the true cooperativity and affinity as measured by intracellular Ca^{2+}, and is only meant as a relative measure of these parameters which cannot be translated directly into intracellular Ca^{2+}-affinities and cooperativities). It is striking that even though the relative increase in mini release produced by KO of Syt1 differs dramatically between excitatory and inhibitory synapses (mIPSCs ~2-fold increase; mEPSCs ~4-fold increase), the apparent Ca^{2+}-affinity and Ca^{2+}-cooperativity in wild-type and Syt1 KO mice are almost identical between the two types of synapses. This, among others, validates the approach.

The results of Fig. 2 answer the first two of the three questions posed above — Ca^{2+}-triggering of spontaneous release is indeed fundamentally different between wild-type and Syt1-deficient synapses, and this is similarly true for excitatory and inhibitory synapses. As regards the third question of the identity of the Ca^{2+}-sensors involved, it is striking that the Ca^{2+}-dependent properties of spontaneous and evoked release in wild-type vs. synaptotagmin-deficient synapses appear to be very similar. Specifically, both spontaneous and evoked release exhibit a lower apparent Ca^{2+}-affinity but a higher apparent Ca^{2+}-cooperativity in wild-type synapses than in synaptotagmin-deficient synapses. Since in cortical wild-type synapses, Syt1 serves as the primary Ca^{2+}-sensor for evoked release, whereas in Syt1 KO synapses an as yet unidentified Ca^{2+}-sensor for asynchronous release takes over this role[27,29], it is tempting to speculate that Syt1 also serves as primary Ca^{2+}-sensor for spontaneous release, and that the unknown asynchronous Ca^{2+}-sensor adopts this role in the Syt1 KO synapses. This hypothesis implies that Syt1 and the asynchronous Ca^{2+}-sensor both act on primed vesicles, and that the former normally clamps the latter (Fig. S3). This hypothesis predicts that the Ca^{2+}-affinity of Syt1 should dictate the magnitude of spontaneous and evoked release in wild-type synapses, a prediction that can be readily tested.

Knockin mutations in Syt1 Ca^{2+}-binding sites

To test the hypothesis that mIPSCs and mEPSCs are triggered by Ca^{2+}-binding to Syt1, we employed mice carrying knockin point mutations in the Ca^{2+}-binding site of the Syt1 C2A-domain[32,33]. Three different mutations were analyzed: D232N, D238N, and R233Q (referred to as D2N, D8N, and R3Q, respectively; Fig. 3a). Previous studies demonstrated that these mutations selectively alter the Ca^{2+}-binding properties of Syt1. Specifically, the D2N-mutation enhances Ca^{2+}-dependent binding of Syt1 to SNARE complexes without altering its phospholipid-binding properties[33], whereas the D8N and R3Q mutations moderately (D8N) or severely (R3Q) decrease the apparent Ca^{2+}-affinity of phospholipid binding by Syt1 without altering SNARE complex binding[32,33]. Moreover, the R3Q mutation was shown to alter the apparent Ca^{2+}-affinity of release correspondingly, an

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observation that provided the formal proof of the function of Syt1 as Ca^{2+}-sensor for release32, whereas the effects of the other mutations on the apparent Ca^{2+}-affinity and Ca^{2+}-cooperativity of release were not tested. Thus, the various knockin mice carrying the three different point mutations in Syt1 provide a tool to test whether Syt1 has equivalent functions as a Ca^{2+}-sensor in evoked and in spontaneous release.

We first measured the effects of the three knockin mutations on evoked release, by comparing neurons from mutant and littermate control mice in the same experiments. We found that the three knockin point mutations caused distinct effects on the apparent Ca^{2+}-affinity of release that correlated with their biochemical effects: the D2N mutation increased the apparent Ca^{2+}-affinity (i.e, decreased the apparent K_d for Ca^{2+}), whereas the D8N and the R3Q mutations decreased the apparent Ca^{2+}-affinity of release (i.e, increased the apparent K_d for Ca^{2+}; Figs. 3b-3d). None of the mutations significantly altered the apparent Ca^{2+}-cooperativity of release, as expected (Fig. S4a). We then measured the effects of the three knockin mutations on spontaneous release, and observed the same result, proving that miniature release is triggered by Ca^{2+}-binding to Syt1 (Figs. 4a-4c and S4b). The increase in mini frequency observed in knockin mice containing the D2N Syt1 mutation that increases its apparent Ca^{2+}-affinity can be fully blocked by preincubation with BAPTA-AM, demonstrating that it conforms to the same overall mechanism (Fig. S5). Thus, in cultured cortical neurons spontaneous release is normally driven by Ca^{2+}-binding to Syt1.

Effect of Syt1 knockin mutations in slices

A potential concern of the experiments described above is that they were performed in cultured neurons where abnormal Ca^{2+}-transients could be generated. To address this concern, we measured the effects of the three knockin mutations on spontaneous mini release in acute brain slices from the Syt1 knockin mice described above, and their littermate wild-type controls (Figs. 5a and 5b). We observed the same effect of the knockin mutations as in cultured neurons, indicating that Syt1 functions as the Ca^{2+}-sensor for spontaneous release in wild-type synapses.

Finally, to investigate whether the role of Syt1 as a Ca^{2+}-sensor for spontaneous release operates during a physiologically meaningful regulation of spontaneous release, we monitored the effect of nicotine on spontaneous release in brain slices from wild-type and knockin mice. Nicotine is known to bind, among others, to presynaptic acetylcholine receptors, and thereby to regulate spontaneous release11-14, as confirmed here for wild-type synapses (Figs. 5c and 5d). In synapses from Syt1 knockin mutant mice, spontaneous release was still increased by nicotine, but in the context of the overall regulation of release by Syt1, i.e., the overall amount of spontaneous release continued to be dictated by the apparent Ca^{2+}-affinity of Syt1 present in the synapses examined (Figs. 5c and 5d). This result suggests that the Syt1-dependent regulation of spontaneous release operates under physiological conditions.

Syt1 Ca^{2+}-binding sites clamp spontaneous release

How does deletion of Syt1 activate, i.e. derepress Ca^{2+}-triggering of mini release at Ca^{2+}-concentrations lower than those that activate Syt1 itself? To explore this question, we

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infected cultured neurons from Syt1 KO mice with control lentivirus, or lentiviruses expressing wild-type or mutant Syt1 in which the Ca^{2+}-binding sites of either the C2A-domain, the C2B-domain, or both C2-domains of Syt1 were mutated (Fig. 6a). In these mutants, three aspartate residues in the respective Ca^{2+}-binding sites of the C2A- and/or the C2B-domain were exchanged for alanine residues, thereby abolishing Ca^{2+}-binding to the C2-domains. The mutant C2-domains are expressed at similar levels in the infected neurons and are still folded well (Fig. S6 and data not shown), allowing us to determine the effect of Ca^{2+}-binding to the individual C2-domains to the Ca^{2+}-triggering and clamping of spontaneous exocytosis.

As expected27, evoked IPSCs were impaired in Syt1 KO neurons, but fully rescued by expression of wild-type Syt1 (Fig. 6b). Mutation of the Ca^{2+}-binding sites of the C2A-domain partially blocked rescue of evoked responses (50% decrease), whereas mutation of the C2B-domain or of both C2-domains completely blocked rescue of evoked release (Fig. 6b). This result is consistent with previous studies showing that Ca^{2+}-binding to both the C2A- and the C2B-domain is involved in the Ca^{2+}-triggering of synchronous release, with the C2B-domain being relatively more important31,34-36. Strikingly, the mutations of the C2-domain Ca^{2+}-binding sites also impaired the ability of Syt1 to clamp spontaneous release (Figs. 6c and S7). However, the relative efficacy of different mutants differed between evoked and spontaneous release, in that the C2A-domain mutation was more deleterious than the C2B-domain mutation for the clamping function of Syt1 (spontaneous mini frequency: C2B-domain mutant = ∼6 Hz, C2A-mutant = ∼10 Hz), whereas the opposite was observed for the release function of Syt1 (Figs. 6c and S7).

To more precisely quantify the similarity of the effect of the C2A- and C2B-mutations on miniature release, we measured the Ca^{2+}-dependence of the mIPSC frequency in Syt1 KO neurons expressing wild-type Syt1 or the C2A- or C2B-domain mutant of Syt1 (Figs. 6d-6f and S8). Quantitation of the apparent Ca^{2+}-affinity and Ca^{2+}-cooperativity of spontaneous release revealed that neither the C2A-nor the C2B-mutation rescued the change in either parameter (Figs. 4d and 4e), despite similar expression levels. These results suggest that in addition to triggering synchronous and spontaneous release, the Ca^{2+}-binding sites of both C2-domains are involved in clamping the asynchronous Ca^{2+}-sensor, with the C2A-domain being more effective than the C2B-domain in clamping the second Ca^{2+}-sensor, whereas the C2B-domain is more effective than the C2A-domain in triggering release.

Ca^{2+}-triggering and clamping of release

A plausible hypothesis to account for the requirement of the C2-domain Ca^{2+}-binding sites for clamping mini release is that these sites clamp mini release in a Ca^{2+}-independent manner, but activate evoked and spontaneous release in a Ca^{2+}-dependent manner. It is likely that the ability of Syt1 to evoke synchronous release requires a close proximity of the Syt1 C2-domains to the membrane where the SNAREs are, because — as confirmed by the experiments on the knockin mutations (Figs. 3-5) — Syt1 functions by simultaneous, rapid, and Ca^{2+}-dependent binding to SNARE-complexes and to phospholipids. Indeed, a most remarkable feature of synaptotagmin-triggered release is the speed with which it operates during an action potential, which can best be accounted for by a lack of conformational

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changes involved, and by short reaction distances. This argument should not apply to the function of Syt1 as a clamp of the asynchronous Ca^{2+}-sensor, or as a Ca^{2+}-sensor for spontaneous release, because these functions do not involve transient Ca^{2+}-fluxes induced by action potentials.

To test these predictions, we examined the effect of increasing the distance of the Syt1 C2-domains from the vesicle membrane on spontaneous and evoked release (Fig. 7a). We expressed Syt1 with a duplicated linker sequence between the C2-domains and transmembrane region. The double-linker mutant rescued only 50% of evoked Ca^{2+}-triggered release, but did not alter the apparent Ca^{2+}-affinity or Ca^{2+}-cooperativity of evoked release as expected, because the C2-domains are not changed (Figs. 7b, 7c, and S9). Moreover, the double-linker mutation did not alter the clamping function of Syt1 (Figs. 7d and 7e), as predicted by the overall hypothesis outlined above. Thus, although both evoked and spontaneous release normally utilize Syt1 as a Ca^{2+}-sensor, only the former requires rapid coupling of the C2-domains to the membrane because it is dependent on the Ca^{2+}-dynamics induced by an action potential, whereas the latter operates via a random sensing of Ca^{2+}-sparks and clamping of the asynchronous Ca^{2+}-sensor.

DISCUSSION

Evoked and spontaneous neurotransmitter release are generally held to represent distinct types of release that are differentially regulated1-3. Their distinc nature is evidenced by the fact that spontaneous release is maintained in the presence of the sodium-channel inhibitor tetrodotoxin, which abolishes action potentials and evoked release. Here we show, however, that despite their differential regulation, these two types of release are mechanistically the same, in that they both are triggered by Ca^{2+}-binding to Syt1 (Figs. 1-5). The major evidence for this conclusion rests on the three Syt1 knockin mutations that we utilized for the present experiments. We previously demonstrated that these mutations either increase Ca^{2+}-dependent binding of Syt1 to SNARE complexes (D2N), or decrease the apparent Ca^{2+}-affinity of Syt1-binding to phospholipids (D8N and R3Q)32,33. We now show in a direct comparison of all three knockin mutations that they cause a corresponding change in evoked release, and a precisely equivalent change in spontaneous release (Figs. 3-5).

The finding that Syt1 functions as Ca^{2+}-sensor for both spontaneous and evoked release was surprising because Syt1 was thought to be physiologically activated only by Ca^{2+}-influx during an action potential, as the Ca^{2+}-affinity of Syt1 is too low to allow it to be activated by submicromolar resting Ca^{2+}-concentrations32. Indeed, it is likely that spontaneous release is triggered not by the resting Ca^{2+}-concentration in the presynaptic terminal, but by Ca^{2+}-sparks 17,18. Ca^{2+}-sparks may arise from internal stores or Ca^{2+}-influx, as indicated by the increase in spontaneous release induced by thapsigargin, nicotine and caffeine, and the dependence of spontaneous release on extra- and intracellular Ca^{2+} (Figs. 1, 2, 5, and S2). The Syt1 knockin mutations caused similar changes in spontaneous release in neurons in culture (Figs. 3 and 4) and in acute brain slices (Fig. 5), suggesting that the role of Syt1 as Ca^{2+}-sensor for spontaneous release is not peculiar to cultured neurons but generally applicable. Moreover, our paper supports previous suggestions7-9 that spontaneous release is physiologically important. Ca^{2+}-regulation generally implies a physiologically controlled

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function; thus, the finding that spontaneous release is controlled by Ca^{2+}-binding to Syt1 implies a physiological role, as is also supported by the observation that the potentiation of spontaneous release by nicotine operates via Ca^{2+}-binding to Syt1 (Fig. 5). Many neurotransmitters and neuromodulators act by increasing or decreasing presynaptical Ca^{2+}-concentrations[10-16], suggesting that these agents may control synaptic circuits, at least in part, by regulating Syt1-dependent spontaneous release without triggering action potentials.

A major argument against the notion that Syt1 and other synaptotagmins are Ca^{2+}-sensors for spontaneous release was the finding that deletions of Syt1 and of Syt2 paradoxically increase spontaneous release[26-28]. We now show that the paradoxical increase in spontaneous release induced by deletion of Syt1 is due to the unclamping not of SNARE complexes, but of an alternative second Ca^{2+}-sensor. This finding argues against the notion that Syt1 acts as a clamp of SNARE-complexes[20,21], a notion that was plausible given the binding of Syt1 to SNARE complexes and the fact that SNARE complexes are likely assembled during priming, ready to go for release[19], but is contradicted by the present data. Clearly, SNARE complexes are tight, and do not simply pop, because otherwise spontaneous fusion would not be Ca^{2+}-dependent.

The second Ca^{2+}-sensor for spontaneous release that is unclamped by the Syt1 KO has a significantly higher apparent Ca^{2+}-affinity and a significantly lower apparent Ca^{2+}-cooperativity than Syt1. These properties correspond to those of the second Ca^{2+}-sensor for release that drives asynchronous exocytosis, and that we previously characterized biophysically in the calyx of Held synapse[37]. In the calyx of Held synapse, Syt2 functions as Ca^{2+}-sensor for synchronous evoked release instead of Syt1, and asynchronous release persists in this synapse after KO of Syt2, similar to asynchronous release in cortical synapses after KO of Syt1[22,28,37]. The calyx of Held synapse, different from other synapses, allows precise manipulations and measurements of presynaptic Ca^{2+}-concentrations and of exocytosis[38,39], permitting us to define the biophysical properties of Syt2 as the Ca^{2+}-sensor for synchronous release, and of the second Ca^{2+}-sensor that mediates asynchronous release, although the identity of this Ca^{2+}-sensor remains unknown[37]. The second Ca^{2+}-sensor exhibits a lower Ca^{2+}-cooperativity than synaptotagmins as Ca^{2+}-sensor for synchronous release, but — because of this lower Ca^{2+}-cooperativity — displays a higher apparent Ca^{2+}-affinity[37]. These findings led to the dual-Ca^{2+}-sensor of neurotransmitter release, according to which evoked release is normally mediated by synaptotagmins, whereas the second Ca^{2+}-sensor generally makes only a minor contribution to release, but is activated during high-frequency stimulation[27,29, 37]. The most parsimonious explanation of our present findings is that the same ‘second’ Ca^{2+}-sensor is also responsible for the increased spontaneous release in Syt1 and Syt2 KO synapses. At present, we cannot rule out the possibility that more than two types of Ca^{2+}-sensors mediate release, i.e., that evoked asynchronous release under physiological conditions and the increased spontaneous release after Syt1 or Syt2 deletions are driven by distinct Ca^{2+}-sensors, but the similarity between the different forms of release argues against this possibility. Thus, it appears likely that the dual-Ca^{2+}-sensor model is generally applicable to spontaneous and evoked release, and that all release is under control of only two competing Ca^{2+}-sensors: synaptotagmins that dominate under physiological conditions, and an

unknown second Ca^{2+}-sensor that kicks in when synaptotagmin is deleted or when synapses are stimulated repeatedly (Fig. S3).

Although it remains unclear how Syt1 and Syt2 clamp the second Ca^{2+}-sensor, our results demonstrate that the Ca^{2+}-binding sites of Syt1 themselves are essential for clamping. Moreover, we show that the C2B-domain of Syt1 is more important than the C2A-domain for evoked exocytosis (Fig. 6b, as reported previously34,35), but that the C2A-domain is more important for clamping spontaneous release than the C2B-domain (Figs. 6c and 6d). Moreover, clamping does not depend on the distance of the C2-domains from the membrane, whereas evoked release does (Fig. 7). Thus, two lines of evidence support the notion that the two functions of Syt1 — Ca^{2+}-sensing vs. Ca^{2+}-clamping — are mediated by related but distinct mechanisms.

Our data also raise new questions. Most importantly, what is the identity of the second Ca^{2+}-sensor, and how does it interact with synaptotagmins? How do the Ca^{2+}-binding sites of Syt1 clamp the second Ca^{2+}-sensor? Since the second Ca^{2+}-sensor operates with an apparently higher Ca^{2+}-affinity, this effect is unlikely to involve Ca^{2+}-binding to Syt1. Clamping must either be mediated by the top C2-domain sequences independent of Ca^{2+}, or by C2-domains with partially occupied Ca^{2+}-binding sites. In the calyx synapse we observed competition between the synchronous and asynchronous Ca^{2+}-sensors37, as did previous studies in hippocampal synapses40,41. In contrast, the present experiments, and our previous studies on cortical synapses27, provide evidence for clamping of asynchronous release by the synchronous Ca^{2+}-sensor. This issue may be related to the puzzling absence of increased mini-release in Syt1-deficient autapses29,32; resolving it will require identification of the asynchronous Ca^{2+}-sensor. Another intriguing question is whether there is a physiological regulation of Syt1 that inactivates it (and evoked release), thereby activating spontaneous mini release, a possibility that would confer a new dynamic dimension onto synapses and account for the physiological role of the mechanisms we describe here. Answering this question again requires identification of the asynchronous Ca^{2+}-sensor.

METHODS

Reagents, mice, and neuronal cultures

APV, CNQX, Bicuculline and CGP55845 were from Tocris, nicotine and caffeine from Sigma, and EGTA-AM and BAPTA-AM from CalBioChem. Neurons were cultured as described22,27 in Modified Eagle Medium (Invitrogen) supplemented with B27 (Invitrogen), glucose, transferrin, fetal bovine serum, and Ara-C (Sigma, St. Louis, MO). Syt1 KO and knockin mice were described previously29,32,33. All experiments were performed either on cortical neurons cultured from littermate wild-type and mutant offspring from heterozygous crossings (for Syt1 knockin mice), or on neurons from the same culture infected with the various test and control viruses (for Syt1 KO mice rescue experiments).

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Generation and use of recombinant lentivirus

The lentiviral constructs used in this paper were based on the pFUW vector (modified from pFUGW vector42), a shuttle vector containing the gene of interest and recombination arms for incorporating into the mammalian genome. The vector includes the HIV-1 flap sequence, the human polyubiquitin promoter-C, a multicloning site, and a WRE element22. Recombinant lentiviruses are produced by transfecting HEK 293T cells with pFUW, pVSVg and pCMV 8.9 using FuGENE. Viruses were harvested 48 h after transfection by collecting the medium from transfected cells, and a 0.45 μm filter was used to remove cellular debris. Protein expression was tested in the transfected 293T cells to ensure viruses were working before they were applied to neurons. Neurons were infected with 500 μl of conditioned cell medium for each 24-well of high-density neurons at 4 days in vitro (DIV), the medium was exchanged for normal growth medium at 6 DIV, and cells were analyzed at 14-18 DIV. Syt1 point mutations were generated using standard procedures43. The Syt1 2 X linker constructs were made by PCR using two sets of primers (JX051: CGGAATTCATGGTGAGTGCCAGTCATCCT and JX065: GGCAGGCACTGCAGAAGGACG; and JX066: GAAAGTATAGGAACTTCGTCGATCGACCTCG and OH05303, GCTCTAGATTACTTCTTGACAGCCAGCATGGC).

Electrophysiological recordings from cultured cortical neurons were performed essentially as described22,33,44. mIPSC and eIPSC were recorded in the presence 10 μM CNQX and 50 μM APV. mEPSC s were recorded in the presence of 30 μM bicuculline and 5 μM CGP55845. EPSCs were recorded with a pipette solution containing (in mM) 123 K-gluconate, 10 KCl, 1 $MgCl_2$, 10 HEPES, 1 EGTA, 0.1 $CaCl_2$, 1 K_2ATP, 0.2 Na_4GTP, and 4 glucose, pH adjusted to 7.2 with KOH. mIPSCs and mEPSCs were analyzed by Mini Analysis Software (search parameters: mIPSC, Gain: 20; Blocks: 3940; Threshold (pA): 10; Period to search for a local maximum (μs): 20000; time before a peak for baseline (μs): 10000; Period to search a decay time: 20000; Fraction of peak to find a decay time: 0.5; Period to average a baseline (μs): 2000; Area threshold: 30; Number of points to average for peak: 3; Direction of Peak: negative; mEPSC, Gain: 20; Blocks: 3940; Threshold (pA): 10; Period to search for a local maximum (μs): 20000; Time before a peak for baseline (μs): 5000; Period to search a decay time: 5000; Fraction of peak to find a decay time: 0.5; Period to average a baseline (μs): 2000; Area threshold: 10; Number of points to average for peak: 3; Direction of peak: negative). The initial analysis was done automatically by the software with subsequent visual proofreading. Application of 0.5 M sucrose (in extracellular solution) was effected with a picospritzer for 30 s (pressure = 10 psi), with puffing pipette tip placed 50 μm from the soma. For Ca^{2+} titration experiments, IPSCs, mIPSCs and mEPSC were recorded in bath solutions containing the various Ca^{2+} concentrations (0+1mM EGTA, 0.2, 0.5, 1, 2, 5, 10 for mIPSC or mEPSC; 0.5, 1, 2, 5, 10 mM for IPSC), and the frequency of mIPSCs or mEPSCs or the amplitude of the evoked IPSCs was plotted as a function of the Ca^{2+} concentration as logarithm axis. The curves were fitted with a Hill function (Y=Vmax*X^n/(K^n+X^n, where Vmax is the maximal response; K is the Ca^{2+}-affinity; n is the cooperativity32), using Igor pro 4.03 software for the fitting, and the maximal responses, the apparent K_d for Ca^{2+}, and the Hill coefficient (i.e. apparent Ca^{2+}-cooperativity) were derived from the fits32.

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Electrophysiological recordings from acute cortical slices

Cortical slices (300 μm) were prepared from male littermate mice aged 13-16 days. Anesthetized mice were decapitated, the brains were removed and placed into ice cold dissection buffer (in mM: 87 NaCl, 3 KCl, 1.25 NaH_2PO_4, 7 $MgSO_4$, 26 $NaHCO_3$, 20 D-glucose, 75 sucrose, 1.3 ascorbic acid, and 0.5 $CaCl_2$), and slices were cut with a Leica Vibratome. The slices were incubated at 34°C in artificial cerebrospinal fluid (in mM: 126 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 25 D-glucose, and 2 $CaCl_2$) gassed with 95%O_2/5%CO_2 for 1 hr, and then kept at room temperature under the same conditions until they were transferred to the recording chamber that was perfused at 2 ml/min with carbogenated artificial cerebrospinal fluid containing 20 μM CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), 50 μM APV and 1 μM TTX to measure mIPSC. Slices were equilibrated for 10 min prior to recordings. All recordings were performed in layer 2/3 pyramidal neurons of the somatosensory cortex; pyramidal neurons were identified by their size and single apical dendrite. Recordings were obtained in voltage-clamp whole-cell mode using a Multiclamp 700B amplifier and a holding potential of -70 mV. The whole-cell pipette solution contained (in mM): 145 KCl, 5 NaCl, 10 HEPES, 10 EGTA, 0.3 Na_2GTP, 4 MgATP, and 10 QX-314. Pipettes used for whole-cell recording had a resistance of 3-5 MΩ; neurons with a series resistance of >20 MΩ or a leak current of >200 pA were discarded. Spontaneous miniature post-synaptic currents were monitored over a 5 minute period, and the series resistance was monitored before and after each recording. Data were analyzed offline using Mini-Analysis. At least three littermate pairs of wild-type and D2N, D8N, and R3Q knockin mice were analyzed for each parameter.

Statistical analyses

All statistical comparisons were made using Student's t-test or a two-way ANOVA as described in Suppl. Table S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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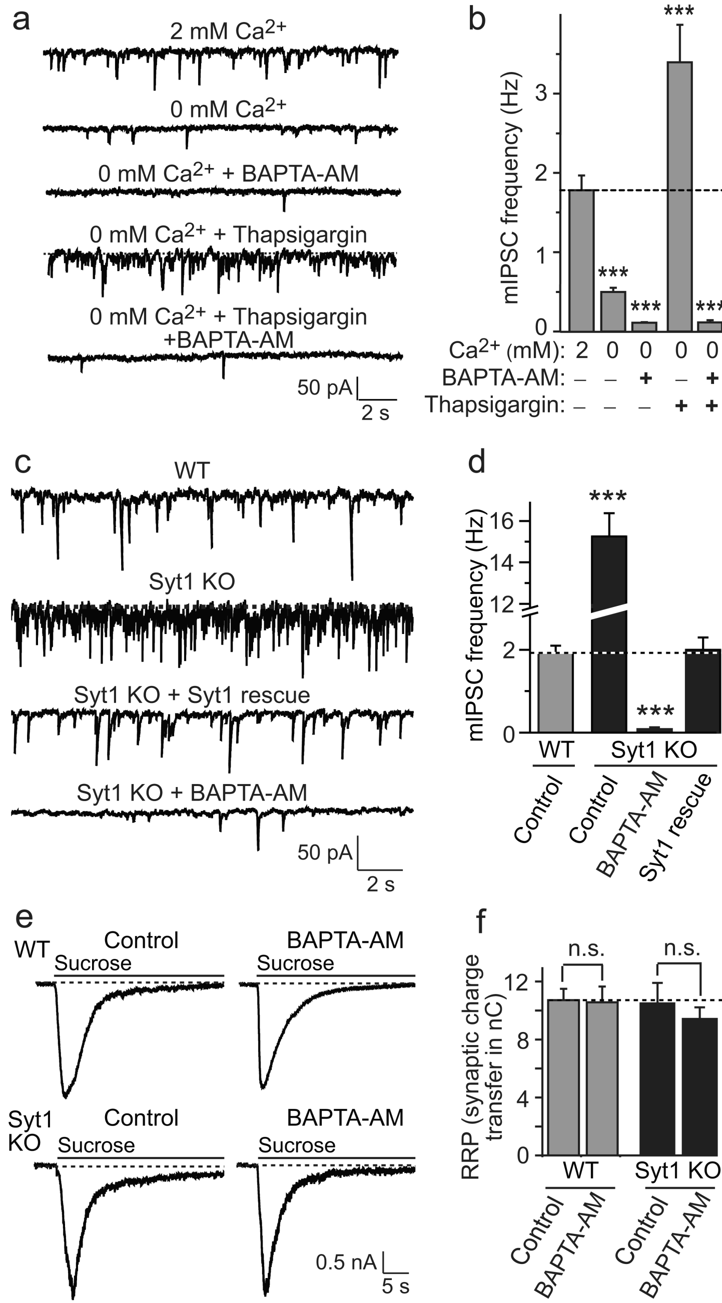

Figure 1. Spontaneous miniature release is Ca^{2+}-dependent in wild-type and Syt1 KO synapses

a.-d. Representative traces (a,c) and summary graphs (b,d) of mIPSCs monitored by whole-cell recordings in cultured cortical neurons from wild-type (WT) and Syt1 KO mice under the indicated conditions (BAPTA-AM: 10 μM applied at 37 °C for 1 hr prior to and during recordings; thapsigargin: 1 μM added five min after stable recordings were established). All recordings from cultured neurons were obtained at 14-16 days in vitro; all mini recordings were produced in 1 μM TTX; mIPSCs were monitored in the presence of 50 μM APV and

10 μM CNQX. Rescue of Syt1 KO neurons was performed by lentiviral infection at 4 days in vitro22.

e, **f.** Representative traces (e) and summary graphs (f) of IPSCs induced by puffing 0.5 M sucrose onto neurons from WT and Syt1 KO mice without or with pretreatment with BAPTA-AM. Recording conditions are the same as above. Summary graphs depict the synaptic charge transfer integrated over 30 s to estimate the size of the readily releasable pool (RRP).

Data shown in panels b,d, and f are means ± SEMs (see Suppl. Table S1 for all numerical values; *** = p<0.001; n.s. = non significant).

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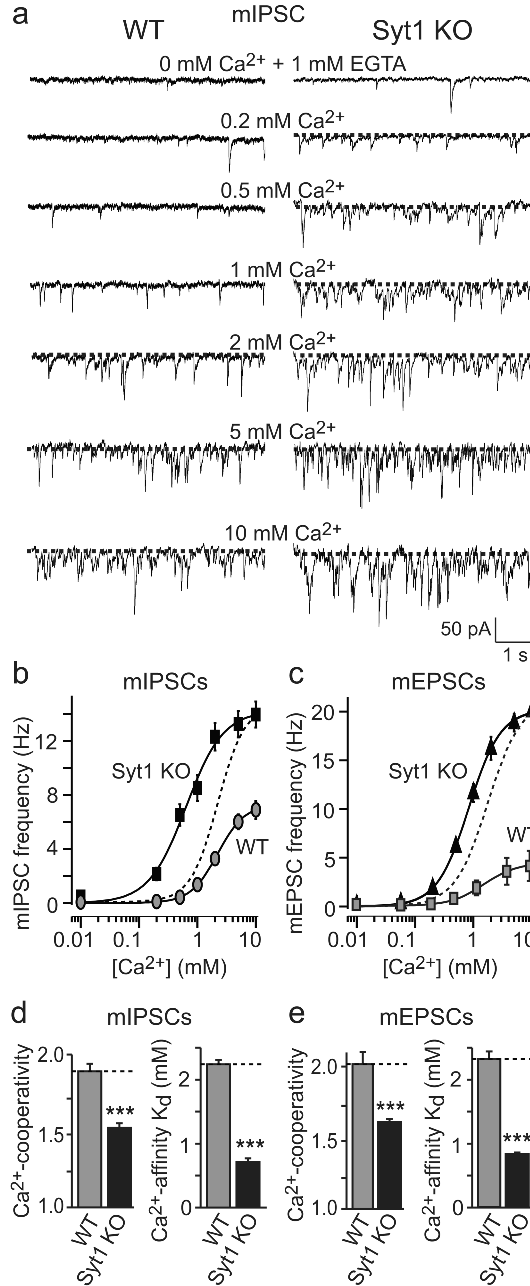

Figure 2. Enhanced frequency, increased apparent Ca^{2+}-affinity, and decreased Ca^{2+}-cooperativity of spontaneous release in Syt1 KO synapses

a-c. Ca^{2+}-dependence of the frequency of mIPSCs and mEPSCs. Panel a exhibits representative mIPSC traces (see Suppl. Fig. S2 for mEPSC traces), and panels b and c summary graphs of the Ca^{2+}-dependence of the mIPSC and mEPSC frequency (continuous lines = Hill function fits; dotted lines = scaled WT Ca^{2+}-dependence).

d, **e.** Mean apparent Ca^{2+}-cooperativity and Ca^{2+}-affinity of mini release at inhibitory (d) and excitatory (e) synapses determined by Hill function fitting to individual experiments.

Data shown in panels b-e are means ± SEMs (see Suppl. Table S1 for all numerical values; *** = $p<0.001$).

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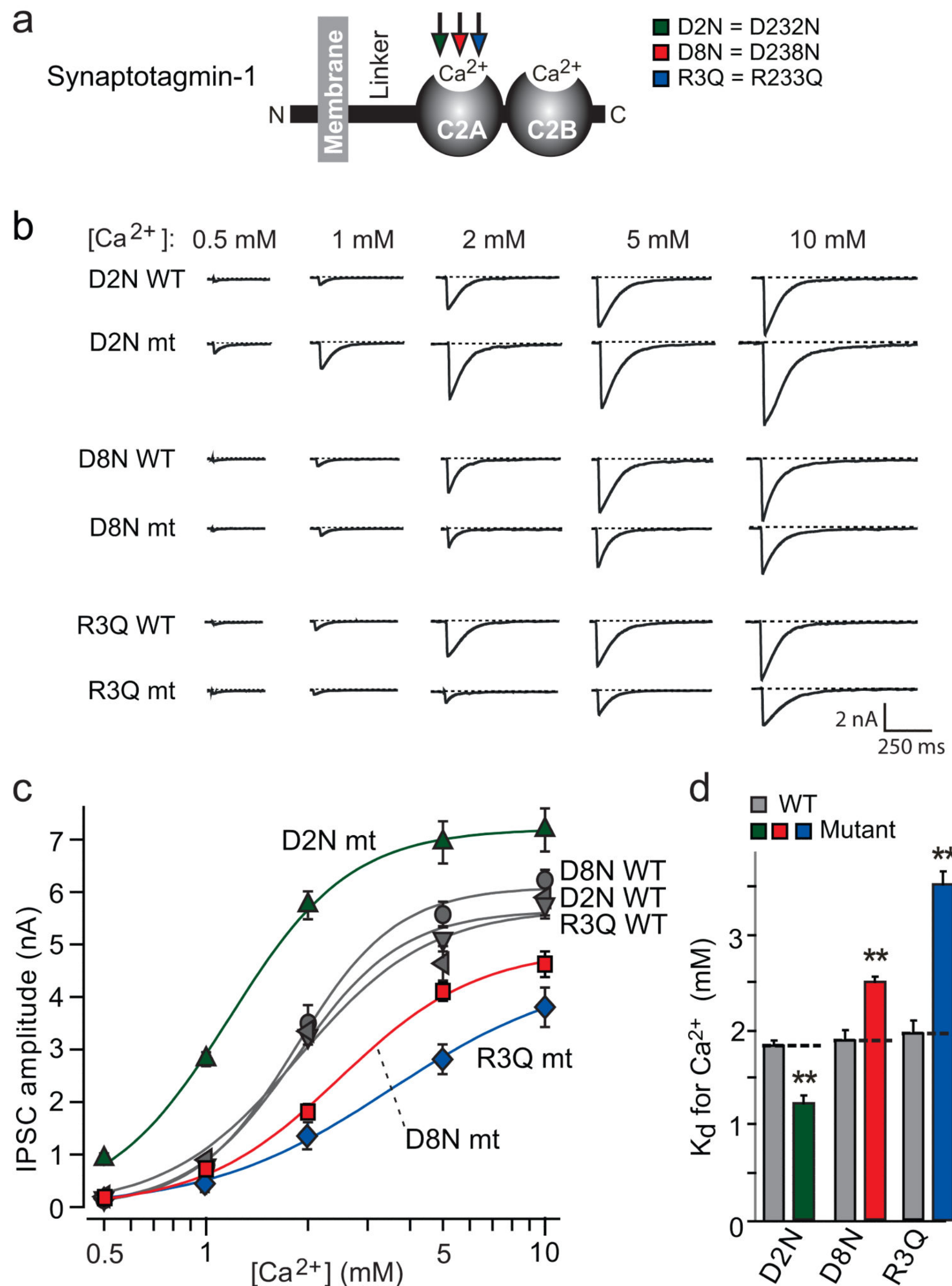

Figure 3. Knockin mutations altering the apparent Ca^{2+}-affinity of Syt1 cause corresponding changes in the apparent Ca^{2+}-affinity of evoked release

a. Point mutations introduced into the C2A-domain of mouse Syt1 by knockin32,33. The D2N-mutation increases Ca^{2+}-dependent binding of Syt1 to SNARE complexes33, whereas the D8N- and R3Q-mutations decrease the apparent Ca^{2+}-affinity of Syt1-phospholipid membrane complexe32,33.

b. Representative traces of evoked IPSCs monitored in cultured neurons from littermate WT and Syt1 D2N, D8N, and R3Q knockin mice.

c. Mean IPSC amplitudes in neurons from WT and knockin mice plotted as a function of the extracellular Ca^{2+}-concentration.

d. Mean K_d for extracellular Ca^{2+} of IPSCs, determined by Hill function fits to individual Ca^{2+}-titration experiments as shown in panels c and d.

In all experiments with knockin mice, each mutant is analyzed with its own separate littermate WT control. Data are means ± SEMs (see Suppl. Table S1 for all numerical values; ** = $p<0.01$; for other parameters, see Suppl. Fig. S3).

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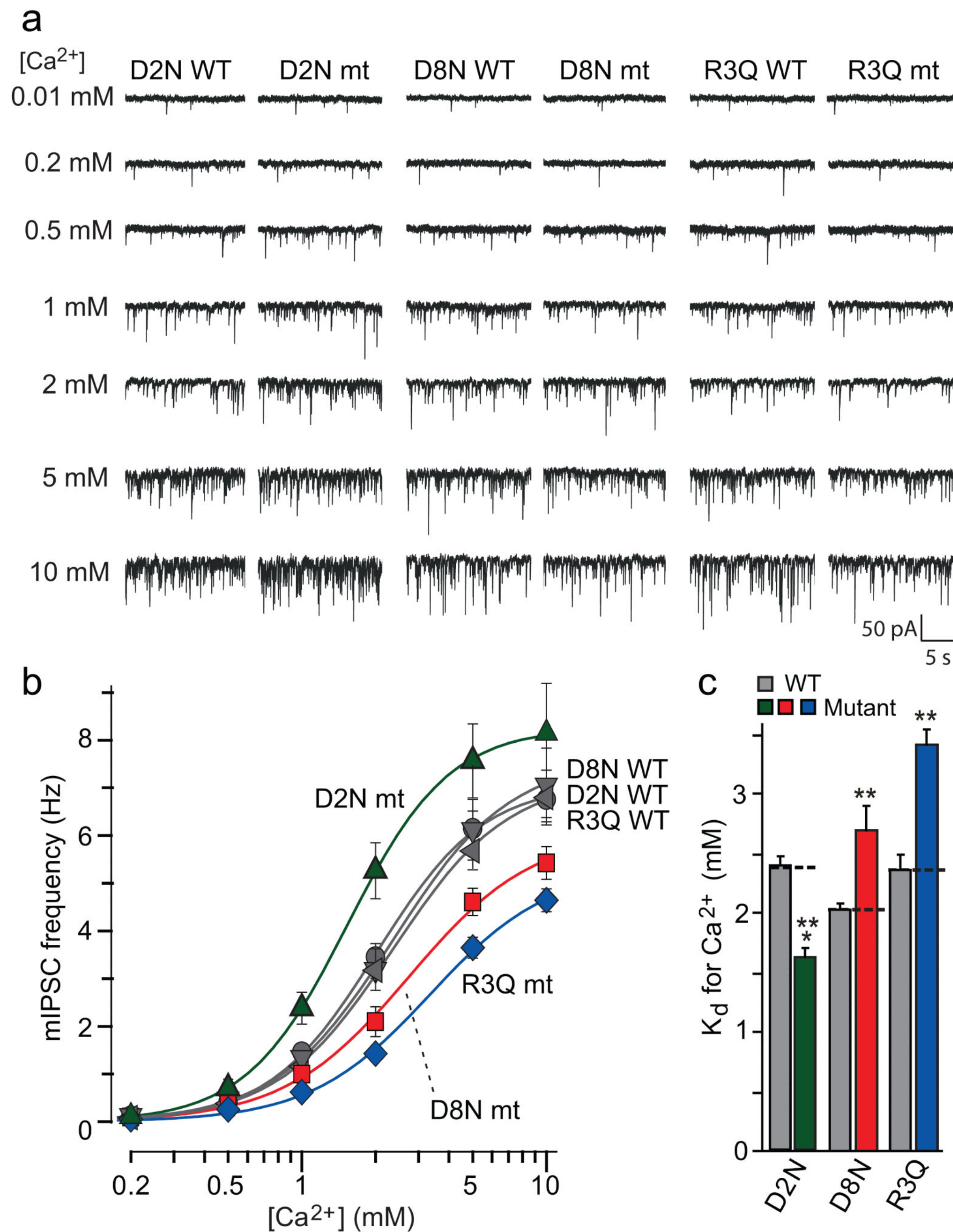

Figure 4. Knockin mutations altering the apparent Ca^{2+}-affinity of Syt1 cause corresponding changes in the apparent Ca^{2+}-affinity of spontaneous release

a. Representative mIPSC traces monitored at different Ca^{2+}-concentrations in neurons cultured from littermate WT and knockin mice with the indicated mutations

b. Mean mIPSC frequency plotted as a function of the extracellular Ca^{2+}-concentration

c. Mean K_d for extracellular Ca^{2+} for mIPSCs as determined by Ca^{2+}-titrations

Data are means ± SEMs (see Suppl. Table S1 for all numerical values; ** = p<0.01; *** = p<0.001).

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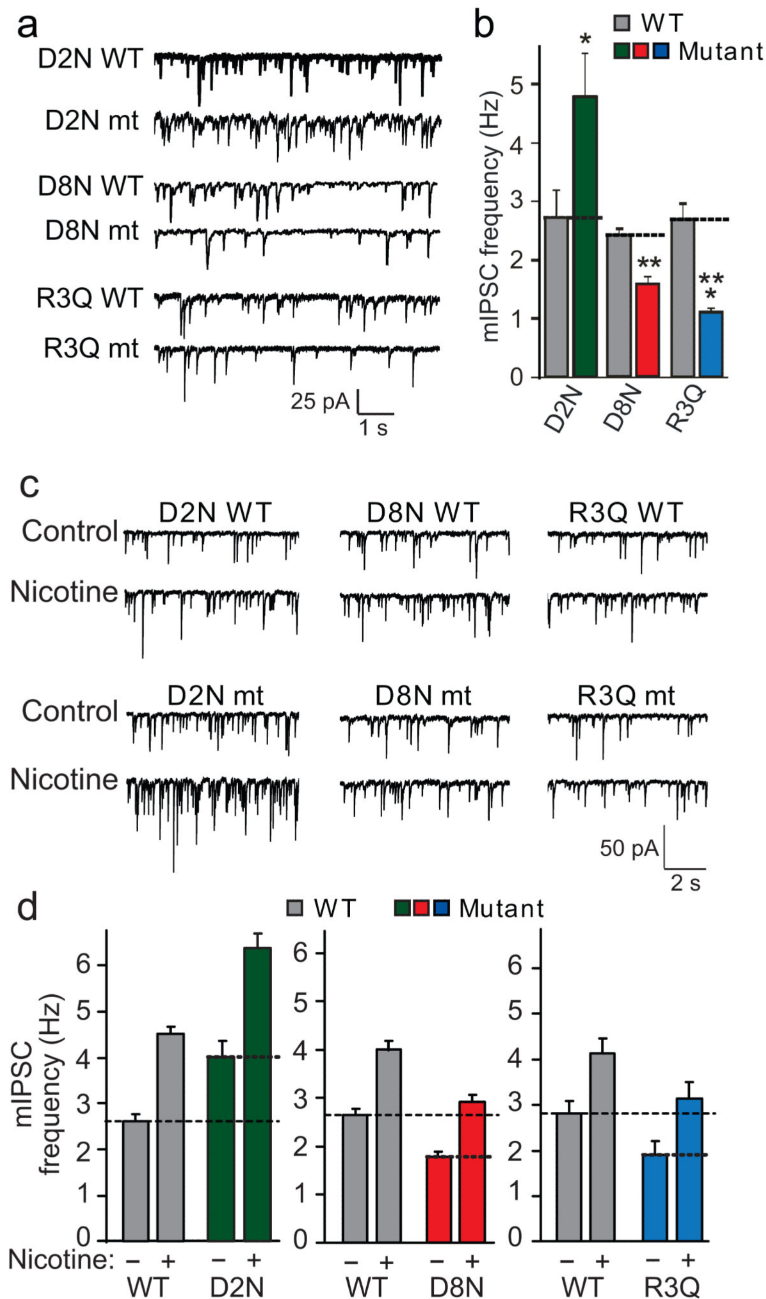

Figure 5. Knockin mutations in Syt1 alter spontaneous release monitored in acute slices

a, **b.** Representative traces (a; calibration bars at the bottom apply to all traces above the bars) and summary graphs (b) of mIPSCs recorded in acute cortical slices from littermate WT and Syt1 knockin mice. Note that the increase frequency of spontaneous release in D2N-mutant mice is fully blocked by BAPTA-AM (Suppl. Fig. 3).

c, **d**. Representative traces (c) and frequency summary graphs (d) of mIPSCs monitored in acute cortical slices WT and Syt1 knockin mice before and after addition of 100 μM

nicotine, applied at a perfusion rate of 2 ml/min at 5 min after stable recordings were established.

Data are means ± SEMs (see Suppl. Table S1 for all numerical values; * = p<0.05; ** = p<0.01; *** = p<0.001).

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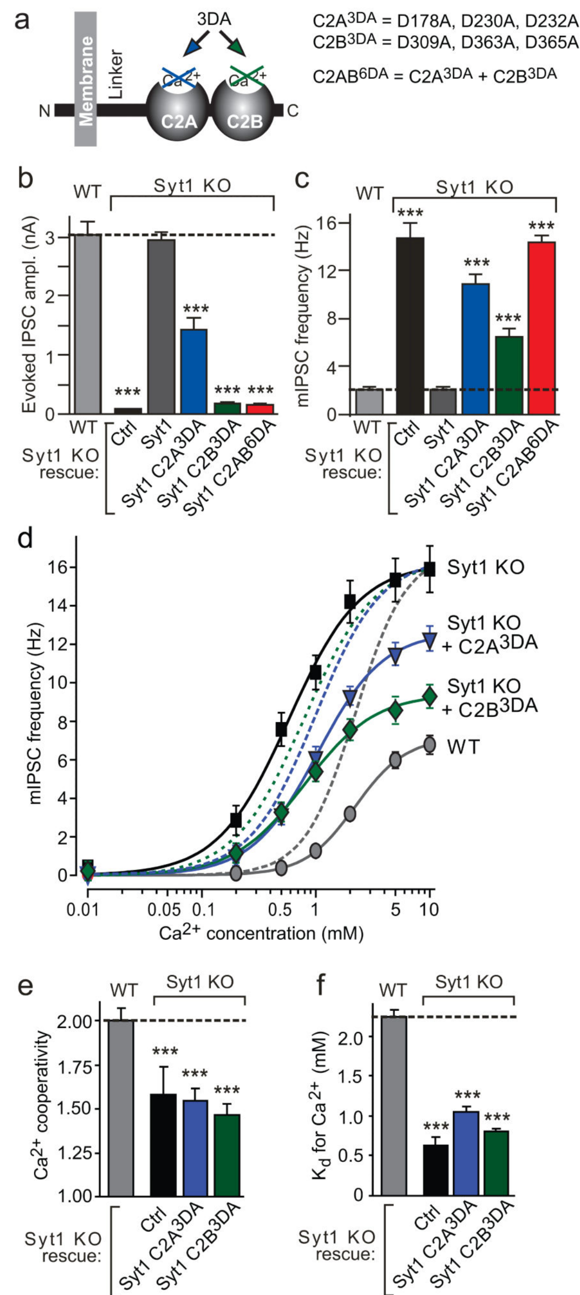

Figure 6. Syt1 clamping of spontaneous release requires intact Syt1 Ca^{2+}-binding sites

a. Diagram of mutations in the C2A- and/or the C2B-domain of Syt1 that abolish Ca^{2+}-binding to individual C2-domains. Each mutation consists of three separate aspartate-to-alanine substitutions in the Ca^{2+}-binding site (hence called DA mutations), with the precise substitutions listed on the right. Mutations were designed based on the atomic structures of the C2A- and C2B-domains40,41.

b, **c**. Comparison of the evoked IPSC amplitude (a) and spontaneous mIPSC frequency (b) in neurons from littermate WT or Syt1 KO mice. Syt1 KO neurons were infected either with

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control lentivirus (Ctrl), or with lentivirus expressing WT Syt1 (Syt1) or mutant Syt1 (Syt1 C2A^{3DA} = mutant lacking the C2A-domain Ca^{2+}-binding sites; Syt1 C2B^{3DA} = mutant lacking the C2B-domain Ca^{2+}-binding sites; Syt1 C2AB^{6DA} = mutant lacking the C2A- and the C2B-domain Ca^{2+}-binding sites; see Suppl. Figs. 4-6 for immunoblots and representative traces).

d. Mean spontaneous mIPSC frequency as a function of the extracellular Ca^{2+}-concentration in WT or Syt1 KO neurons expressing C2A^{3DA}- or C2B^{3DA}-mutant Syt1 (continuous lines = fittings with Hill functions; dotted lines = scaled fittings; see Suppl. Fig. 6 for representative traces).

e, **f.** Summary graphs of the mean Ca^{2+}-cooperativity (e) and K_d for extracellular Ca^{2+} (f) determined from the experiments shown in panel d.

Data shown are means ± SEMs (see Suppl. Table S1 for all numerical values; *** = $p<0.001$; n.s. = non-significant).

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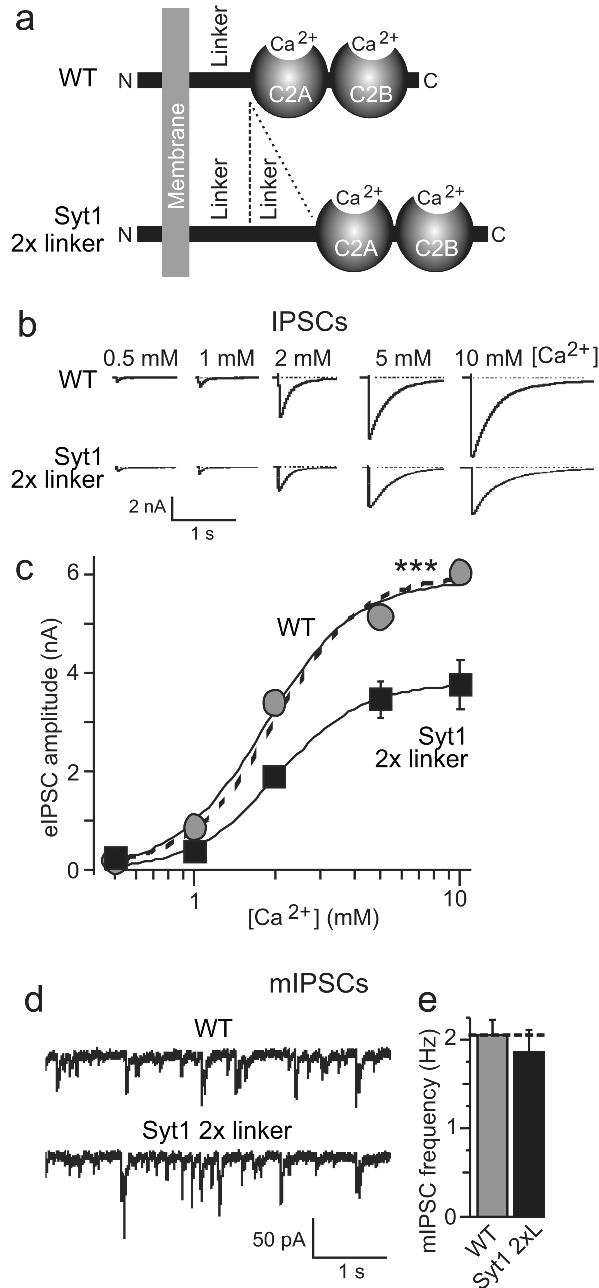

Figure 7. Membrane proximity of Syt1 C2-domains contributes to evoked but not spontaneous release

a. Diagram of insertion mutation in Syt1 that duplicates the linker separating C2-domains from the membrane.

b. Representative traces of evoked IPSCs in WT neurons and Syt1 KO neurons expressing mutant Syt1 with a double linker. Responses were monitored at different extracellular Ca^{2+} concentrations

c. Plot of the mean IPSC amplitude as a function of the extracellular Ca^{2+}-concentration. See Fig. S9 for summary graphs.

d, **e.** Representative traces (e) and summary graph of the frequency (f) of mIPSCs monitored in WT neurons and in Syt1 KO neurons expressing the double-linker mutant of Syt1.

Data shown in all panels are means ± SEMs (see Suppl. Table S1 for all numerical values; *** = $p<0.001$; n.s. = non-significant).

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